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Dual-colour imaging with GFP variants

Jan Ellenberg, Jennifer Lippincott-Schwartz and John F. Presley

Green fluorescent protein (GFP) has become an important tool in cell biology and is widely used as a reporter for imaging intracellular proteins and structures in live cells. Recently, spectral variants of GFP with red- and blue-shifted fluorescence emissions have been characterized, opening the possibility of double labelling with two different-coloured GFP fusion proteins. This article reviews recent advances in this technique, with special emphasis on time-lapse imaging applications in living cells.

Green fluorescent protein (GFP) is a protein produced by the jellyfish *Aequorea victoria* that contains a fluorescent cyclic tripeptide whose fluorescence is preserved in chimeric fusions, made using recombinant DNA techniques[1,2], that can be expressed in many organisms. As a result, GFP has become an important fluorescent tag for studying intracellular proteins that are otherwise technically difficult or impossible to observe in live cells with the light microscope. GFP chimeras have been used to monitor cell migration[3,4], trafficking between membrane-bound organelles[5–8], movement of signalling proteins between cytosolic and membrane-bound pools[9,10] and many other cellular processes.

Until recently, it was not possible to label more than one protein at a time using GFP since there were no suitable variants that fluoresce at different wavelengths. Double and triple labelling of cells was therefore limited to immunofluorescence methods in fixed cells and to experiments using fluorescent dyes or injection of proteins labelled *in vitro*. In each case, different markers are directly attached to easily distinguishable fluorophores (e.g. rhodamine and fluorescein) and can be used to determine colocalization or noncolocalization of intracellular structures. However, there are now spectrally distinguishable variants of GFP that can readily be used for double-label experiments[11–14] and that offer enormous potential for biological and medical research in living cells. In this article, we describe GFP pairs and techniques that can be used for double labelling experiments as well as their most important advantages and disadvantages. We then present examples of double-label GFP experiments and discuss future prospects for multicolour *in vivo* labelling of proteins.

Double labelling strategies with GFP

GFP variants differing in their excitation and emission spectra have been created by directed mutagenesis of the Ser-Tyr-Gly sequence that makes up the fluorophore and of amino acids that interact with the fluorophore[11–13]. Initially, Yang and coworkers described a scheme to partially separate wild-type GFP from a redshifted variant (S65G based, 'rsGFP4')[15]. Heim and coworkers demonstrated that three GFP variants [blue (Y66H based, 'P 4-3'), cyan (Y66W based, 'W7') and green (S65T)[16] could label three populations of bacteria and that they had distinct colours[13]. Rizutto *et al.* demonstrated

TABLE 1 – DUAL-COLOUR GFP IMAGING LITERATURE SURVEY[a]

Ref.	GFPs	Application	Pros and cons (+/−)
15	wtGFP (green), rsGFP4 (green)	Single double-label images of *E. coli* and CHO cells expressing the variants separately	−wtGFP is dim and photobleaches rapidly, separation poor since the setup for blue excitation detects both variants equally well
13	P 4-3 (blue), W7 (cyan), S65T (green)	One triple-label image of *E. coli* cells expressing the variants separately	+First demonstration of three distinguishable colours of GFP −Blue variant is dim and photobleaches rapidly; no quantitation of the significant crossover
17	P 4-3 (blue), wtGFP (green)	Single double-label image of HeLa cell coexpressing nucleoplasmic and mitochondrially targeted GFP variants	+Clean separation −Blue photobleaches rapidly, lack of brightness; requires high expression levels
18	P4-3 (blue), S65T (green)	High-resolution 3-D reconstruction of mitochondria and ER in a single living HeLa cell	+Clean separation allows high-quality 3-D reconstruction in live cells −Blue photobleaches rapidly and is dim; requires very sophisticated imaging setup and high expression levels
19	W2 (cyan), S65T (green)	Time-lapse imaging of *Dictyostelium* cells expressing the variants separately	+Variants are very photostable and can be imaged simultaneously −Significant crossover of cyan emission in the green channel requires quantitative image processing. Requires a customized CLSM
21	sg50 (blue), sg25 (green)	Single double label image of 293 cells coexpressing HIV-1 Rev and Gag fusions	+Clean separation using standard CLSM configuration −Blue (although optimized) photobleaches rapidly and is dim
23	W7 (cyan), 10C (yellow)	Time-lapse imaging of COS7 cells coexpressing GFP variants targeted to the Golgi complex and nuclear envelope	+Variants are very photostable and can be separated without significant crossover with simple microscope setup −Images have to be acquired sequentially[b]

[a]Abbreviations: +, pro; −, con; CLSM, confocal laser scanning microscope; *E. coli*, *Escherichia coli*; GFP, green fluorescent protein; 3-D, three dimensional; wt, wild-type.
[b]Sequential image acquisition can be circumvented on CLSM systems that can switch excitation lasers line-by-line using acoustic optical tunable filters.

the use of a blue fluorescent protein (BFP) and wtGFP/S65T to take single double-label image sets of mitochondria in living cells[17] and recently to reconstruct interactions between the endoplasmic reticulum (ER) and mitochondria three-dimensionally[18]. Finally, in a first time-lapse imaging application, Zimmermann and coworkers demonstrated that a very similar cyan variant to W7 ('W2'[13]) and S65T could be used to track cells expressing one or the other GFP variant during the formation of a *Dictyostelium* slug and fruiting body[19] (see also Table 1).

The two basic strategies for double labelling, BFP–S65T and cyan–S65T, are fundamentally different, and it is instructive to consider the advantages and disadvantages of the two methods. The use of BFP and S65T [or improved commercial variants (Table 2) such as EBFP, EGFP[20] and BFP, rsGFP[21] – of which the latter pair has recently been used to localize HIV Rev and Gag[21]] is conceptually no more difficult than the separation of DAPI/Hoechst and fluorescein performed routinely for immunofluorescence studies. Each GFP variant has its own complete filter set composed of three components – an excitation filter, a dichroic beamsplitter and an emission filter. A fluorescein set suffices for S65T (although better filter sets have been described[22]), while UV illumination and blue emission filter adequately captures BFP fluorescence[13]. Crossover (or fluorescence visible through the 'wrong' filter set) is minimal (Fig. 1b). Unfortunately, BFP (including 'improved' varieties) is very dim and photobleaches extremely rapidly[20,23]. This has made it unsuitable for time-lapse acquisition of high-resolution images at any reasonable protein expression level.

Zimmermann and coworkers[19] devised an interesting scheme for using the fluorescein-like S65T and the more photostable cyan variant W2 (which differs from BFP in having purple excitation and cyan emission) as a double-label pair. They took advantage of the fact that the two GFPs can be excited together using a confocal microscope and the 457-nm line of an argon laser. The emissions are then split using a dichroic beamsplitter and sent to two different detectors. This allows simultaneous imaging of the two GFPs. Unfortunately, the emission spectrum of W2 is quite broad (very similar to W7 in Fig. 1a) and significant fluorescence inevitably appears in the S65T channel. This crossover must then be computer corrected postexperimentally, which is not practical for long time-lapse

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TABLE 2 – SOME GFP VARIANTS USED IN DOUBLE LABELLING AND THEIR NOMENCLATURE[a]				
Variant name	**Ex/em**	**Amino acid changes**	**Commercial availability**	**Ref.**
P 4-3	381/445	Y66H, Y145F	No	13
BFPsg50/BFP	387/450	F64L, Y66H, V163A	Q	21
EBFP	380/440	F64L, S65T, Y66H, Y145F[c,d]	C	20
W2	432/480	Y66W, I123V, Y145H, H148R, M153T, V163A, N212K	No	13
W7	434/474	Y66W, N146I, M153T, V163A, N212K[b]	No	13
ECFP	434/474	K26R, F64L, S65T, Y66W, N146I, M153T, V163A, N164H N212K[c,d]	C	14
S65T	489/511	S65T	C	16
GFPsg25/rsGFP	473/509	F64L, S65C, I167T, K238N	Q	21
EGFP	488/507	F64L, S65T[c,d]	C	20
10C	514/527	S65G, V68L, S72A, T203Y	No	12
EYFP	514/527	S65G, V68L, S72A, T203Y[c,d]	C	14

[a]Abbreviations: Ex, excitation peak; Em, emission peak; GFP green fluorescent protein. Suppliers: C, Clontech Inc., Palo Alto, CA, USA; Q, Quantum Biotechnologies, Montreal, Canada. **Boldface** indicates GFP variants particularly useful for double labelling in mammalian cells having improved folding properties at 37°C as well as humanized codon usage ('enhanced' variants, E_FP) or mRNA stability (rsGFP and BFP).
[b]Has the Q80R mutation that does not change the spectrum.
[c]Variant has one valine residue inserted at position 2, which is not counted.
[d]Variant has the H231L mutation that does not affect the spectral properties.

sequences (a detailed description of crossover correction followed by quantitative analysis is in Ref. 24). The primary disadvantage of this scheme is that it requires a custom-configured confocal microscope as well as some image-processing expertise, making it inaccessible to many laboratories.

The use of W7/ECFP and 10C/EYFP as a double-label pair

We have found that W7[13] or its improved and much brighter variant ECFP[14] makes an excellent double-label pair with 10C[12] (identical fluorescence spectrum to EYFP[14], which is also commercially available) and can be separated easily and cleanly (crossover ≤3%) using conventional fluorescence microscopes[23]. This separation relies merely on the differing excitation spectra of the two GFP variants (Fig. 1a) and therefore requires changing only an excitation filter (which can be done under computer control on many microscopes). Since both W7/ECFP and 10C/EYFP are photostable, it is possible to acquire time-lapse sequences of more than one hundred images at reasonable (or even low) protein expression levels. Configuring an existing microscope provided it is already equipped with an electronic detector (such as a cooled CCD camera) and a computer-controlled excitation filter wheel requires only the purchase of filters with a total cost of less than a thousand dollars. Specifications of the required filter sets can be found in Ref. 23.
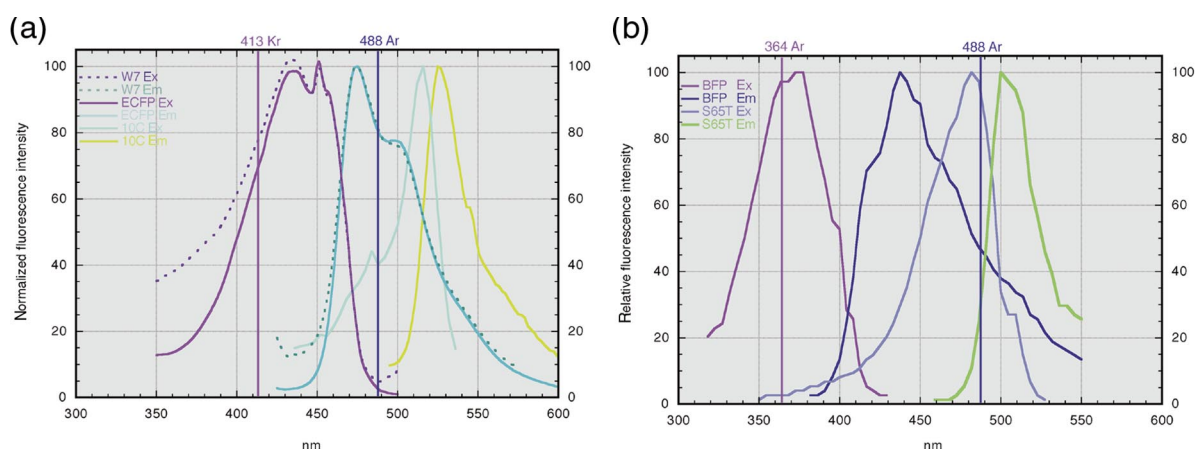
FIGURE 1

Comparison of green fluorescent protein (GFP) variant fluorescence spectra. (a) Fluorescence spectra of W7/ECFP (excitation: purple dotted and solid lines, respectively; emission: cyan dotted and solid lines) and 10C (excitation: light cyan line; emission: green–yellow line). Differences between W7 and ECFP are most likely due to higher background in the W7 sample. (b) Spectra of blue fluorescent protein (BFP; P 4-3/excitation: purple line; emission: dark blue line) and S65T (excitation: light blue line; emission: green line). Laser lines for excitation using a confocal laser scanning microscope are indicated. Fluorescence intensities were normalized for comparison and acquired as described in Ref. 23.

Configuring a confocal laser scanning microscope (CLSM) for double label with these GFP variants is more difficult. 10C/EYFP is readily excited using the 488-nm line of a conventional argon laser (used on most CLSMs to excite fluorescein) and even better with the 514-nm line of some argon ion lasers, but standard lasers designed for CLSM use generally do not have appropriate laser lines (between 400–425 nm) for exciting W7/ECFP without inappropriate crossover excitation of 10C (Fig. 1a). This problem can be overcome by using a krypton laser with a 413-nm line (Coherent Inc.[23]).

We have made various 10C/ECFP chimera pairs for proteins involved in membrane trafficking and nuclear envelope assembly/disassembly (Ref. 23; J. Ellenberg and J. F. Presley, unpublished). Fig. 2a shows confocal images of a COS7 cell where the nuclear envelope was labelled with lamin B receptor–10C (LBR–10C) and the Golgi apparatus with galactosyl transferase–W7 (GT–W7). We are currently using ECFP rather than W7 since it has identical spectral properties for double-label experiments (see Fig. 1) and is much brighter. Fig. 2b shows an example of a COS7 cell in which chromatin is labelled with histone 2B–ECFP (H2B–ECFP[25]) and the nuclear envelope is again labelled using LBR-10C. We have also been able to image membrane-transport intermediates containing the vesicular stomatitis virus G protein[6] or the KDEL receptor[26] tagged with ECFP with both the CLSM and by conventional microscopy (J. F. Presley, unpublished).

Fig. 3 shows time-lapse images of an NRK cell labelled with LBR–10C and H2B–ECFP. The initial image shows the cell at prometaphase, with histone labelling clearly visible on condensing chromosomes, while the LBR–10C is dispersed throughout the ER[27]. As the cell progresses through mitosis, the nuclear envelope reforms and the chromosomes decondense. With our confocal microscope, it has been possible to acquire sequences of several hundred successive double-label pairs[23]. In acquiring confocal sequences such as these, both fluorophores are imaged without using UV light. Such imaging conditions appear to be harmless to the cells as can be seen by the completion of mitosis (a process highly sensitive to photodamage[28]).

A disadvantage of this strategy for double labelling with 10C/ECFP (on both confocal and conventional microscope systems) is that the image pair is acquired sequentially rather than simultaneously. This is unimportant for studies of processes such as mitosis in which events unfold over many minutes. It creates difficulties, however, when structures are observed that move significant distances each second (such as membrane-

transport carriers moved by microtubule motors) or fast events such as diffusional fluorescence recovery after photobleaching (FRAP, see related article in this issue by White and Stelzer[29]). However, on some confocal microscopes that use acoustic optical tunable filters to modulate the excitation lasers, this problem can be solved by switching lasers line by line within a single frame scan. We also have found that illumination levels must be kept low on our conventional microscope system to avoid damaging the cells – probably because our W7/ECFP excitation bandpass filter passes some ultraviolet radiation to the specimen. This problem should be avoidable in the future by using an extremely narrow bandpass above 400 nm to exclude UV excitation.

Towards a brighter future

Double labelling with spectral variants of GFP is a young and rapidly developing field. As new GFP mutants become available and with innovations in imaging and microscopy technology, future systems to achieve *in vivo* double labelling are bound to improve and change the techniques described in this article. Two recent developments deserve note as they might lead to tagging and imaging three or even more proteins at a time in a single living cell,
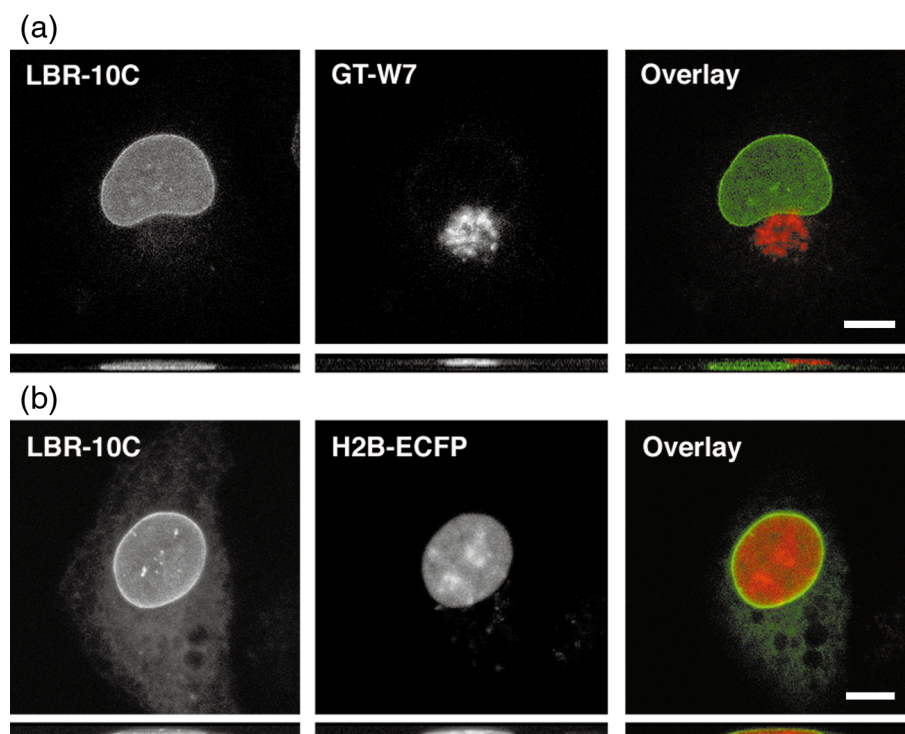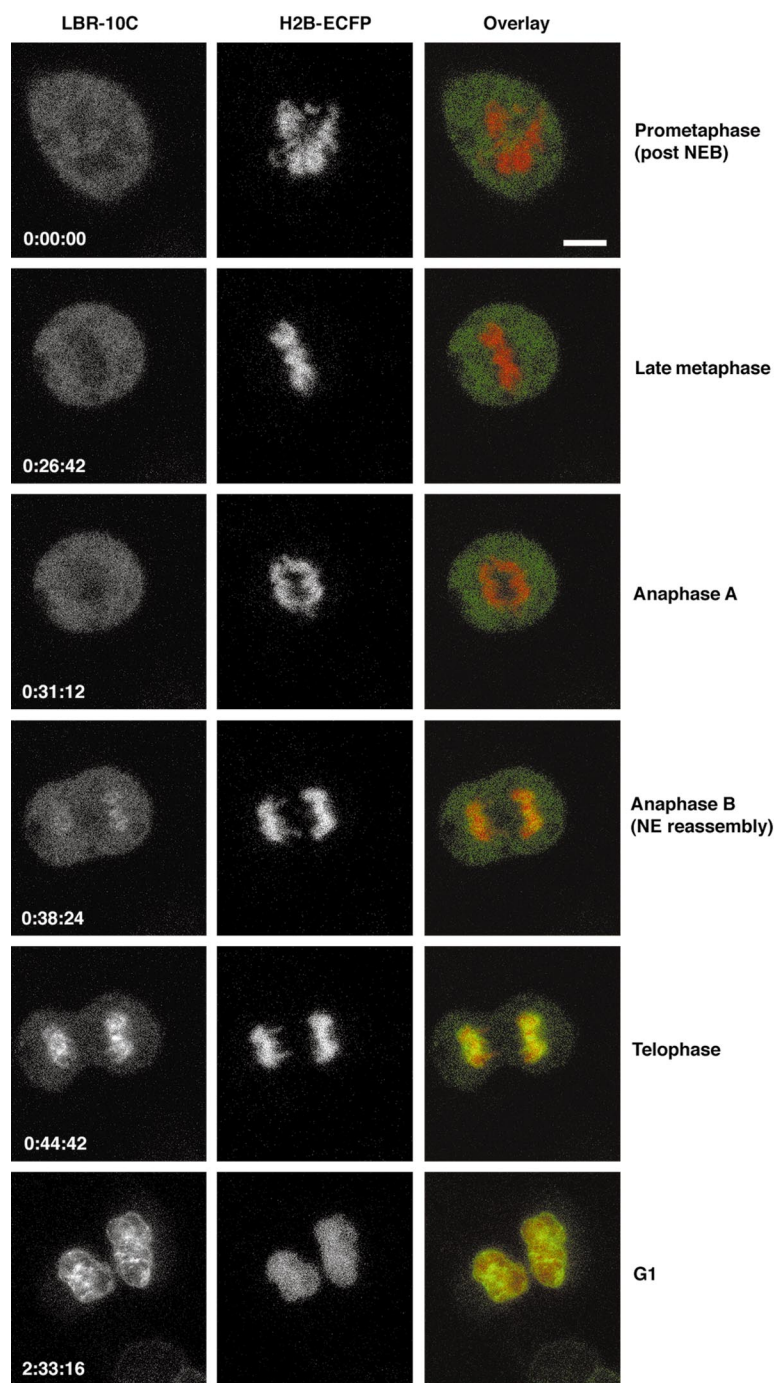
FIGURE 2

Single double-label image pairs using W7/ECFP and 10C variants of green fluorescent protein (GFP). (a) Confocal double-label image pair of live COS7 cells coexpressing the nuclear envelope marker lamin B receptor fused to the 10C variant of GFP (LBR–10C, left panel) and the Golgi complex marker galactosyltransferase fused to the W7 variant of GFP (GT–W7, middle panel). Golgi membranes can be seen around the microtubule-organizing centre close to the nuclear membrane. (b) Similar image pairs of live NRK cells coexpressing LBR–10C (nuclear envelope, left panel) and the chromatin marker histone 2B fused to ECFP (H2B–ECFP, middle panel). The nuclear envelope, which shows a few invaginations from the upper and lower surface of the membrane, surrounds the intranuclear interphase chromatin. Images are projections of z-stacks at 0° (en face view, upper row) and 90° (side view, lower row), respectively, and were acquired as described in Ref. 23. Bars, 10 μm.

LBR-10C **H2B-ECFP** **Overlay**

Prometaphase (post NEB) — 0:00:00

Late metaphase — 0:26:42

Anaphase A — 0:31:12

Anaphase B (NE reassembly) — 0:38:24

Telophase — 0:44:42

G1 — 2:33:16

FIGURE 3

Double-label time-lapse imaging of nuclear envelope reassembly. Time-lapse sequence of live NRK cell coexpressing the nuclear envelope marker lamin B receptor fused to the 10C variant of green fluorescent protein (LBR–10C, left column) and the chromatin marker histone 2B fused to ECFP (H2B–ECFP, middle column) and merged images (right column); each row represents one timepoint of the same cell. The sequence starts in prometaphase after the nuclear envelope has broken down (NEB) and is equilibrated with the endoplasmic reticulum (ER) while the chromatin is still condensing. ER and chromatin are clearly separated from metaphase to anaphase transition (26:42 and 31:12, no yellow in the merged image) and only in anaphase B do nuclear membranes emerge from the ER around the chromatin (38:24, yellow appears in the merged image). In telophase (44:42), nuclear membranes have formed a convoluted shell around the chromatin, which becomes spherical and smoother as cells enter G1 phase (2:33:16). The *in vivo* colocalization of chromatin and nuclear envelope membranes allows a precise cell-cycle timing of events in nuclear envelope reassembly[27]. At each time point (every 54 s), a single image pair was collected sequentially with 9 s delay from the midpoint of the 10C image to the midpoint of the ECFP image. Bar, 10 μm.

as well as supplementing or even replacing current GFP technology.

Murphy and Lagarias[30] have described the use of phytochromes, a family of biliprotein photoreceptors from plants, as fluorescent tags. These proteins, when expressed in cells incubated with the fluorescent cofactor precursor phycoerythrobilin, covalently bind the cofactor and acquire a red rhodamine-like fluorescence that should be easily distinguishable from GFP. In principle, this protein, when combined with two GFPs, should allow triple-label experiments.

In a different approach to tagging proteins *in vivo*, Griffin and coworkers[31] have designed a hexapeptide containing four carefully spaced cysteines that can bind specifically to an arsenic derivative of fluorescein in the presence of small vicinal dithiols. This hexapeptide is a much smaller tag than GFP and is more comparable in size with epitope tags commonly used to facilitate localization of proteins in fixed cells by conventional immunofluorescence. It is likely that fluorophores such as rhodamine or DiI that are easily distinguishable from GFP will eventually also be used to tag proteins in this manner.

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